AC 09/5ED 303

PCT

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) **International Patent Classification** [6]:
G01N 33/68, C07K 1/04 // G01N 33/86

A1

(11) **International Publication Number:** **WO 97/42507**

(43) **International Publication Date:** 13 November 1997 (13.11.97)

(21) **International Application Number:** PCT/GB97/01228

(22) **International Filing Date:** 2 May 1997 (02.05.97)

(30) **Priority Data:**
9609262.2 2 May 1996 (02.05.96) GB

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(81) **Designated States:** JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) **Title:** PEPTIDE ARRAY AND METHOD

(57) **Abstract**

A method of studying a functional protein comprises providing an array of support-bound oligopeptides with different oligopeptides bound in an immunoreactive state at different locations on the support wherein each oligopeptide has defined amino acid residues at two chosen positions and a mixture of amino acid residues at other positions; applying the functional protein in solution to the array; and identifying support-bound oligopeptides that bind to the functional protein. Secondary oligopeptide libraries are used to further elucidate the amino acid sequence of the target protein. The method is used to elucidate the core residues responsible for the binding of a monoclonal antibody to its antigen β-factor XIIa.

PEPTIDE ARRAY AND METHOD

Elucidation of the interactive residues of a functional protein with its peptide ligand provides important information for understanding and manipulating the biological processes in which the functional protein is involved. Combinatorial peptide libraries are powerful tools for identifying these interactive sequences. Libraries comprising large numbers of peptides ranging from 10^6 - 10^{12} are available for this purpose. But such libraries are necessarily complex and expensive.

Combinatorial peptide libraries have also been built up on beads by a "split-mix" approach, which ensures that each bead carries only one sequence. A difficulty with this approach is that the identity of the peptide sequence on a particular bead is not known and must be determined by analysis. Such analysis is time consuming and may be at the limits of available technology.

A "one step" positional scanning approach to identify a peptide ligand has been reported (see R A Houghton *et al*, Biotechniques, 13(6), 901-5). In this technique, a library was made up with peptide mixtures each with an amino acid defined at one position and the other positions containing a mixture of 19 amino acids. H M Geysen describes in US Patent 5,194,392 a corresponding technique in which each oligopeptide of a library contains known amino acid residues at two specified positions. When contacted with a functional protein in solution, only those peptides with the correct amino acids in each position gives the strongest recognition signal. However, individual oligopeptides of each library were mounted on beads or on pins, which is disadvantageous since it requires the use of a rather large amount of functional protein in solution. Also, it is difficult to ensure that each oligopeptide of the library is

paper. The support may be activated, either to provide carboxyl groups for reaction with amine groups of amino acids, or more preferably to provide amine groups for reaction with carboxyl groups of amino acids. For example, a support may be reacted with ethylene diamine to provide primary amine groups. To reduce any problems of steric hindrance, a linker molecule may be provided between the support and the first amino acid residue of each oligopeptide. Thus for example an unnatural amino acid such as β-alanine may be provided on the surface for reaction with the first amino acid of each oligopeptide member of the array. Techniques for synthesising oligopeptides on a support are well known and do not form part of this invention. Preferably the amino acid residues of each oligopeptide of the library, at all positions other than the two chosen positions, are mixtures of all natural L-amino acids except cysteine. Such arrays are readily generated by combinatorial chemical methods.

The support is preferably planar. Alternatively the individual locations of the support may be the individual wells of a microtitre plate. The array may comprise (19 x 19) 361 different oligopeptides bound at 361 different locations on the support. The oligopeptide at each different location would comprise a different combination of two defined natural L-amino acid residues excluding cysteine which may be the same or different.

Although different oligopeptides of the array may have different lengths, it will usually be convenient for all oligopeptides of the array to be the same length. This should be great enough to permit effective binding to an epitope of the functional protein, that is to say generally comprising at least five or six amino acid residues. Longer oligopeptides may lose flexibility or develop structure of their own which make them less able to bind to epitopes of the target functional protein. Preferred length is 5 to 12, e.g. 7 to 9, amino acid residues.

The positions of the two known amino acid residues in these

possible. According to a preferred strategy, this step is performed by constructing several secondary libraries of support-bound oligopeptides in which: each support-bound oligopeptide of each secondary library has the same amino acid residues at the same two chosen positions as the first support-bound oligopeptide; each support-bound oligopeptide of a secondary library has a defined amino acid residue at another chosen position; and the said other chosen position is different for each secondary library.

The target functional protein in solution is applied to each of these secondary libraries. This permits identification of a secondary support-bound oligopeptide in each secondary library that binds to the functional protein. Each secondary support-bound oligopeptide carries a known amino acid residue at a known position. From the information collected from all these secondary library experiments, it is possible to build up a substantially complete picture of an immuno dominant epitope of a (hypothetical) receptor that binds to the target functional protein.

Alternatively, other strategies are possible at this secondary stage of the method. For instance, secondary libraries of support-bound oligopeptides can be prepared in series, rather than in parallel, with the oligopeptides of each library containing one or more known amino acid residue at a known position than the oligopeptides of the previous library. Alternatively a secondary library of oligopeptides on beads can be prepared by a "split-mix approach", provided that the oligopeptide on each bead carries the two defined amino acid residues at the two positions chosen in the first stage.

Preferred membranes based on polyvinylidenedifluoride have several advantages. They are easily derivatised to enable oligopeptides to be stably bound through the N-terminus, in a form which is immunoreactive with a target functional protein in solution. When used in this way, the membrane is found to have very low background noise, so that the

20 mins when the coupling was complete, the membrane was washed three times with 20 ml amine-free N,N-dimethylformamide (Fluka, UK) and the unreacted amino groups were blocked by acetylation with 4%(v/v) acetic anhydride in N,N-dimethylformamide for 10 minutes. The protecting Fmoc group was then removed from the β-alanine with 20 ml of 20%(v/v) piperidine (Fluka, UK) in N,N-dimethylformamide for 5 mins at room temperature after which the membrane was washed 5 times with 20 ml N,N-dimethylformamide. To monitor the efficiency amino-acid coupling the membrane was stained with 0.01%(w/v) bromophenol blue solution in N,N-dimethylformamide for 5 minutes before the addition of the activated amino-acid. The stained membrane was washed three times with 20 ml methanol and finally dried under a stream of cool air. During the formation of the peptide bond the colour of the spot changed from blue to yellow-green. Free amine was removed from the solvents used for peptide bond formation by storing them over molecular sieve type 4A.

Construction of the peptide libraries.

The libraries were constructed on two sheets of derivatised membrane. On the first membrane (1 x 12 cm) a 19 x 19 matrix of peptide mixtures was constructed making a total of 361 groups of peptides. Positions 2 and 4 of the peptide sequence contained defined amino acids, while at each of the remaining positions was added a mixture of the 19 natural amino acids (except cysteine) in equimolar proportions. Having established the amino acids in positions 2 and 4 which gave the best antibody binding in the first scan these were then used in a second scan. The peptides in this scan were synthesised using the so-called "positional scanning approach", but incorporating in positions 2 and 4 the amino acids identified in the first scan. In the second scan one of the 19 natural amino acids was incorporated into a third position while at the remaining 5 positions in the octapeptide was added a mixture of all the amino acids.

dichloromethane and then with N,N-dimethylformamide (20 ml x 3) followed by methanol (20 ml x 3) and after drying was stored at 4°C.

Immunoblotting for locating recognition residues.

Before probing the peptides with the antibody, the membrane was wetted with 20 ml methanol and transferred into 20 ml 0.01 M phosphate buffered saline pH 7.4 with 0.02% (w/v) Tween-20 for three minutes. The membrane was blocked in 20 ml 10% (v/v) new-born calf serum in 0.01 M phosphate buffered saline with 0.02% (w/v) Tween-20, pH 7.4 for two hours. The membrane was washed with (3 x 20 ml) 0.01 M phosphate buffered saline pH 7.4 containing 0.02% (w/v) Tween-20. The appropriate concentration of ascites fluids or purified IgG, containing the murine monoclonal antibody 201/9 against human β-factor XIIa was incubated with the membrane for 2 hour at room temperature. The membrane was washed and finally incubated with an optimised anti-mouse IgG-peroxidase conjugate in 20 ml of 0.01 M phosphate buffered saline pH 7.4 containing 0.02% (w/v) Tween-20 for one hour at room temperature. After extensive washing with 0.01 M phosphate buffered saline with 0.02% (w/v) Tween-20, pH 7.4 and then buffer (3 x 20 ml) without Tween-20, the membrane was subjected to signal development. The membrane was first blotted on tissue paper and rinsed in an enhanced chemiluminescent solution (0.05 M borate buffer pH 8.5 containing 0.4 mM 4-Iodophenol (Fluka, UK) and 1.25 mM Luminol (Sigma, UK) and 2.7 mM H_2O_2 (Sigma, UK)) for 30 sec. The membrane was wrapped with cling film (Lakeland Plastics, UK) and exposed to a light sensitive film, Hyperfilm (Amersham, UK) in a light enhance cassette (CAWO, Germany) for different times depending on signal intensity. The films were developed in Develop-74 (Kodak UK) for 2.5 mins at room temperature and 5 minutes in a fixer solution (Kodak UK). The film was extensively washed with water and dried in the air. The film with the image of the spots was scanned with

and the signal was amplified by an enhanced chemiluminescent assay. The results showed that the spots with the strongest recognition contain Phe and Gln or Ile and Ile in positions 2 and 4 respectively. The sequences of the peptides and the strength of recognition is presented as a grey scale (Fig. 1).

Since each recognised spot was composed of a calculated 4.7×10^7 (19^6) peptides and only the amino acid at positions 2 and 4 were defined, a further series of iterative experiments is required to identity all the residues involved in the binding of the antibody. For this, it would be necessary to incorporate each of the 19 amino acids at the six remaining six positions in the octapeptide sequence. To reduce the number of iterative syntheses a positional scanning technique was adopted. The two amino acids, identified in the first screening library, were incorporated together with a defined amino acid in a third position into the new libraries. The two best recognised peptide mixtures (Fig.1) were chosen for further analysis. These contained in the defined positions 2 and 4 either Phe and Gln in the first peptide or Ile at both positions in the second. To identify the remaining residues of the epitope 12 positional peptide libraries, differing only in the location of the defined position, were synthesised on two sheets of membrane. On the first membrane, the peptides were synthesised in six rows containing nineteen spots each and except for Phe at position 2 and Gln at position 4, the amino acid in each of the six remaining positions was changed in successive rows. Thus, in row one, a total of nineteen peptide mixtures were synthesised and in each of the mixtures, position one was defined as one of 19 amino acids and positions 2 and 4 contained Phe and Gln respectively, all the other positions contained a mixture of the 19 amino acids. In row two, another 19 group of peptide mixtures were synthesised in which position 3 was a single amino acid and as in row one positions 2 and 4 contained Phe and Gln and a mixture of 19 amino acids was used in the remaining positions. The process was repeated until all

suggests that either at position seven and eight the amino acid requirement is less specific than at the other positions, or more likely, that the epitope does not include these residues and that the immunodominant epitope for the monoclonal antibody 201/9 is the sequence Ser-Phe-Leu-Gln-Glu-Asn.

The array was re-used 20 times without any deterioration in sensitivity and specificity.

DISCUSSION

The membrane based peptide libraries provides a rapid and convenient technique to study the molecular interaction of a functional protein with its ligand. In addition, the residues involved in the interaction can be defined without any knowledge of the primary structure. The peptide libraries were synthesised on a piece of hydrophilic polymer membrane which showed very little non-specific protein binding. Typically, only 3-4 days are required for the synthesis of the octapeptide libraries by an operator with little experience of peptide chemistry. The total number of peptides required in the first scan is 361 and in the second scan 114 and since all the peptides in each scan are present on one piece of membrane only a small amount of ligand (antibody) is required for screening. Furthermore, the libraries on the membrane could be regenerated at least twenty times without a noticeable decrease in sensitivity thus enabling the same peptides to be probed by many different ligands.

A "one step" positional scanning approach to identify a peptide ligand has been described. In this technique, the libraries were made up of peptide mixtures each with an amino acid defined at one position and the other positions containing a mixture of 19 amino acids. Only those peptides with the correct amino acid in each position gave the strongest recognition signal. However, in this procedure, since only one residue in the sequence of the octapeptide library was defined, there would

various target proteins bound were identified by probing with a streptavidin/peroxidase conjugate, and the bound conjugate was located using a chemiluminescent assay. Preliminary work had shown that biotinylation of the antibodies and the inhibitor had no effect on their activity.

In a second experiment (results not shown) a scanning array was prepared which contained a series of overlapping oligopeptides based on the amino acid sequence of the inhibitor. This array was probed with β-factor XIIa and the bound β-factor XIIa was detected using a specific monoclonal antibody followed by anti-murine IgG:peroxidase conjugate β-factor XIIa.

FIGURE LEGENDS

Fig. 1 The binding intensity of the peptide mixtures in the libraries for the monoclonal antibody 201/9.

The strength of binding of the monoclonal antibody 201/9 for the peptide mixtures with defined amino acids at positions 2 and 4 read as a 256 grey scale is plotted against the peptide composition. X indicates the positions containing the mixtures of amino acids.

Figs. 2a-f All the amino acids of the epitope identified in a one step positional scan of the libraries.

The vertical axis represents the binding intensity on a 256 grey scale for the recognised peptide mixtures with defined amino acids. The horizontal axis indicates the defined amino acid in the position defined by the formulae:- (Fig 2a) O_1-Phe-X-Gln-X-X-X-X; (Fig 2b) X-Phe-O_3-Gln-X-X-X-X; (Fig 2c) X-Phe-X-Gln-O_5-X-X-X; (Fig 2d) X-Phe-X-Gln-X-O_6-X-X; (Fig 2e) X-Phe-X-Gln--X-X-O_7-X; (Fig 2f) X-Phe-X-Gln-X-X-X-O_8. Where O is the single amino acid listed along the bottom of the figure and X denotes a mixture of all the amino acids.

bound oligopeptide of a secondary library has a defined amino acid residue at another chosen position; and the said other chosen position is different for each secondary library; applying the functional protein in solution to each secondary library and identifying a secondary support-bound oligopeptide in each secondary library that binds to the functional proteins; and using the information generated to identify the peptide sequence of an immudominant epitope of the functional protein.

6. A method as claimed in any one of claims 1 to 5, wherein the array comprises 361 different oligopeptides bound at 361 different locations on the support.

7. A method as claimed in any one of claims 1 to 6, wherein the support is a polyvinylidenedifluoride membrane.

8. An array of support-bound oligopeptides, with different oligopeptides bound in an immunoreactive state at different locations on the support, wherein each oligopeptide has defined amino acid residues at two chosen positions.

9. An array as claimed in claim 8, wherein each oligopeptide has defined amino acid residues at two chosen non-adjacent positions.

10. An array as claimed in claim 9, wherein each oligopeptide is support-bound through its C-terminus and the defined amino acid residues are at the 2- and 4-positions counted from the C-terminus.

11. An array as claimed in any one of claims 8 to 10, wherein the amino acid residues of each oligopeptide, at each position other than the two chosen positions, are mixtures of all natural L-amino acids except cysteine.

12. An array as claimed in any one of claims 8 to 11, comprising 361 different oligopeptides bound at 361 different locations on the support.

13. An array as claimed in any one of claims 8 to 12, wherein the support is a polyvinylidenedifluoride membrane.

Fig.2.

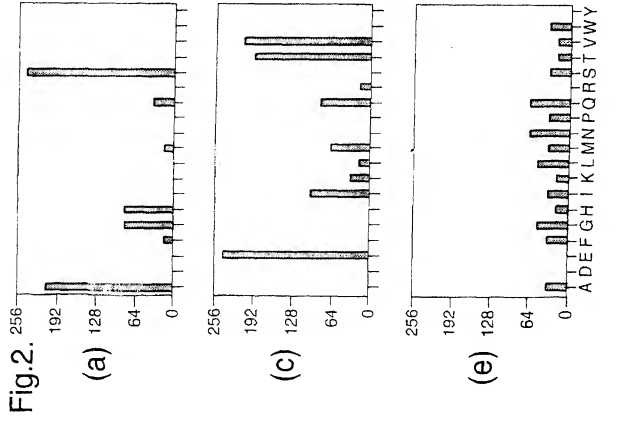
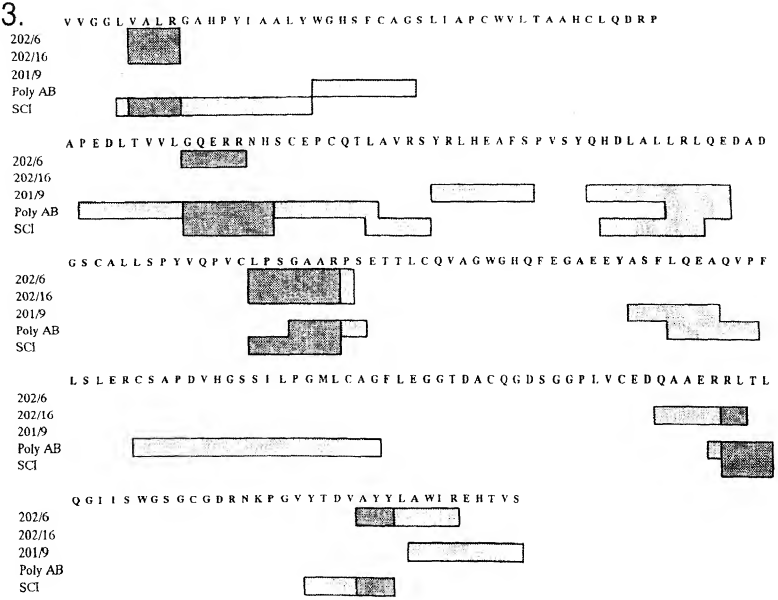


Fig.3.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